METHOD OF ANALYZING PANCREATIC β-CELL AMOUNT AND/OR PANCREATIC β-CELL FUNCTION AND UTILIZATION THEREOF

FIELD OF THE INVENTION

The present invention relates to a method of analyzing pancreatic β-cell amount and/or pancreatic β-cell function, and particularly to an analyzing method by which the amount of pancreatic β-cell and/or the function of pancreatic β-cells can be monitored in real time, and use of such methods.

BACKGROUND OF THE INVENTION

Diabetes mellitus is a medical disorder characterized by abnormal metabolism of sugar, protein, and lipid, resulting from defective secretion of insulin by the insulin-producing cells (β cells) in the pancreas, or defective action of insulin in target cells. The cause of the disorder involves malfunction of more than one gene, along with environmental factors such as infection, obesity, and lack of exercise. Characteristic symptoms shared by the disorder include excessive intake of fluid, excessive discharge of urine, and weight loss.

In Japan, the number of patients diagnosed with diabetes mellitus has been increasing every year. In the 1990s, the figure topped several millions, making up about 5% to 10% of adults over 40 years of age. For clinical purposes, diabetes mellitus is broadly classified into two predominant forms: Insulin-dependent diabetes mellitus (Type 1 Diabetes Mellitus, IDDM); and non-insulin-dependent diabetes mellitus (Type 2 Diabetes Mellitus, NIDDM).

Patients with insulin-dependent Type 1 Diabetes Mellitus absolutely require insulin injection for survival. Type 1 Diabetes Mellitus is characterized by autoimmune destruction of pancreatic β cells initiated against antigens that are induced viral infection, stress, or some other

factors. The onset of Type 1 Diabetes Mellitus therefore involves a significantly reduced level of insulin production. Type 1 Diabetes Mellitus most commonly occurs in adolescents and young adults under age 25. In Japan, Type 1 Diabetes Mellitus appears in about 3% of patients suffering from some form of disease.

Non-insulin dependent Type 2 Diabetes Mellitus covers all forms of diabetes mellitus except for the insulin-dependent form and some unique types. The cause is multiple and involves genetically defective secretion or action of insulin, which leads to a reduced suppressing action for the release of sugar in the liver, and reduced sugar intake in the muscle and fat tissues, and which also leads to increased production of fatty acid, glycerol, amino acid, and sugar intermediate metabolites, with resulting gluconeogenesis in the liver and increased production of fats and ketones. This causes hyperglycemia, ketosis (ketonemia), and hypercholesterolemia.

Hyperglycemia involves discharge of renal sugar, osmotic diuresis, and dehydration when the blood sugar level exceeds the absorption capability of the kidneys. Thus, if hyperglycemia persists for extended time periods, denaturation of the blood vessel wall or narrowing of the blood vessel lumen occurs. This encourages sorbitol metabolism in the cells, which leads to retinopathy,

nephrosis, peripheral neutopathy, myocardinal infarction, brain infarction, and other complications in the blood vessel.

That is, the direct cause of Type 1 Diabetes Mellitus is the absolute loss of function of the pancreatic β cells due to large-scale cell death. In Type 2 Diabetes Mellitus, a reduction in the number of pancreatic β cells, which may be innate or acquired, is associated with the onset and development of the disease.

As described thus far, diabetes mellitus and insulin are closely associated with each other, and the only organ that produces and secretes insulin is the pancreas, or more specifically, pancreatic β cells. Thus, for the treatment of diabetes mellitus, there is a long awaited need for the development of regenerative treatment for the pancreatic β cells, wherein the number of pancreatic β cells is recovered (increased), or the function of pancreatic β cells is improved. Currently, the development of regenerative treatment for the pancreatic β cells is still on the grounding stage; nevertheless, there have been numerous studies and reports concerning functional analysis of pancreatic β cells, regulatory mechanism of gene expression, and the connection between oxidative stress and diabetes mellitus (for example, see Non-Patent Publications 1 and 2). In this regard, there have been

reports concerning a plurality of transcription factors that activate expression of pancreas-specific-genes, and are associated with the development and differentiation of the pancreas (see Non-Patent Document 3, for example).

[Non-Patent Document 1]

Hideaki Kaneto et al., "Beneficial Effects of Antioxidants in Diabetes. -Possible Protection of Pancreatic β-Cells Against Glucose Toxicity-", DIABETES, VOL.48, p2398-p2406, DECEMBER 1999

[Non-Patent Document 2]

Yoshitaka KAJIMOTO, *"Analysis of Expression Regulatory Mechanism for Pancreatic beta cell-specific gene"*, Diabetes, 43(3): 179-181, 2000

[Non-Patent Document 3]

Yoshitaka KAJIMOTO, Yoshimitsu YAMASAKI, Shoji HORI, *Pancreatic Morphogenetic Genes and Abnormalities*, G. I. Research vol.9 no.3, p57 (241) to p64 (248), 2001

As mentioned above, for the treatment of diabetes mellitus, there is a need for the development of therapy and medicaments, etc. for the pancreatic β cells, wherein the number (or amount) of pancreatic β cells is increased, or the function of pancreatic β cells is restored. However, no technique has been developed that analyzes, in real time, whether the amount of pancreatic β cells has increased or the function of pancreatic β cells has

recovered—a technique necessary for the development and evaluation of such therapy.

In a conventional technique of diagnosing or evaluating diabetes mellitus, fluctuations of pancreatic β cell count (or amount) are observed in a pancreas removed from a corpse. However, no technique has been available that analyzes, in real time, fluctuations of pancreatic β cell count (or amount) or the function of pancreatic β cells in living organisms.

In this connection, for the development of therapy or medicaments for diabetes mellitus, there is a strong demand for a method that conveniently and accurately analyzes, in real time, fluctuations of pancreatic β cell count (or amount) or the function of pancreatic β cells *in vitro*, *ex vivo*, or *in vivo*.

The present invention was made to solve the foregoing problems, and an object of the invention is to provide a method, and use thereof, that analyzes, in real time, fluctuations in the number or amount of pancreatic β cells, or the function of pancreatic β cells *in vivo*, *ex vivo*, or *in vitro*.

SUMMARY OF THE INVENTION

The inventors of the present invention diligently worked to solve the foregoing problems. In accomplishing

the invention, the inventors constructed a plasmid construct in which a reporter gene that encodes human secreted alkaline phosphotase (SEAP) is placed under the control of a promoter of transcription factor pdx-1 that activates expression of a pancreatic-specific-gene, and produced transformed cells or transgenic animals that have incorporated the plasmid construct. It was found as a result that the expression level of pdx-1 could be analyzed in real time, using SEAP activity as an index. Based on this finding and the fact that the expression level of pdx-1 increases with increase in the amount of pancreatic β cells, the inventors accomplished the present invention that can evaluate and analyze the amount and/or function of pancreatic β cells in real time.

Specifically, the present invention provides industrially useful methods and substances as defined below.

(1) An analyzing method comprising analyzing an amount and/or function of pancreatic β-cells, using a biological sample that has incorporated therein a recombinant expression vector in which a reporter gene is placed under the control of a promoter region of a pancreatic β-cell-specific gene.

(2) An analyzing method as set forth in (1), comprising:

a transforming step of introducing the recombinant expression vector into the biological sample;

a detecting step of detecting a product of the reporter gene expressed in the biological sample to which the recombinant expression vector was introduced in the transforming step; and

an analyzing step of analyzing an amount and/or function of pancreatic β-cells based on a result of detection performed in the detecting step.

(3) An analyzing method as set forth in (2), wherein, when the reporter gene is a gene that encodes an extracellular secreted product, the detecting step further comprises:

an extracting step of extracting an extract from the biological sample to which the recombinant expression vector was introduced in the transforming step; and

an extract detecting step of detecting the product of the reporter gene included in the extract obtained in the extracting step.

(4) An analyzing method as set forth in any one of (1) through (3), further comprising an expression vector constructing step of constructing the recombinant expression vector.

(5) An analyzing method as set forth in any one of (1) through (4), wherein the recombinant expression vector

includes an enhancer region.

(6) An analyzing method as set forth in any one of (1) through (5), wherein the pancreatic β-cell-specific gene comprises at least one gene selected from the group consisting of pdx-1 gene, NeuroD1 gene, Nkx2.2 gene, Nkx6.1 gene, Pax4 gene, Pax6 gene, insulin gene, glucokinase gene, GLUT2 gene, and amylin gene.

(7) An expression vector in which a reporter gene is placed under the control of a promoter region of a pancreatic β-cell-specific gene.

(8) A transformant to which the recombinant expression vector set forth in (7) is introduced.

(9) An analyzing kit for performing an analyzing method set forth in any one of (1) through (6).

(10) An analyzing kit as set forth in (9), including at least one substance selected from the group consisting of:

(a) a recombinant expression vector in which a reporter gene is placed under the control of a promoter region of a pancreatic β-cell-specific gene;

(b) a transformant to which the recombinant expression vector of (a) is introduced;

(c) a reagent for introducing the recombinant expression vector of (a) into an animal cell; and

(d) a reagent for detecting a product of the reporter gene of (a).

(11) A screening method for screening for a candidate substance of an anti-diabetic drug, comprising:

an administering step of administering a test substance to a biological sample that has incorporated therein a recombinant expression vector in which a reporter gene that encodes an extracellular secreted product is placed under the control of a promoter region of a pancreatic β-cell-specific gene;

a detecting step of detecting the product of the reporter gene that is expressed in the biological sample that was administered with the test substance in the administering step;

an analyzing step of analyzing an amount and/or function of pancreatic β-cells based on a result of detection in the detecting step; and

a determining step of determining that the test substance is a candidate substance of an anti-diabetic drug, when a result of analysis in the analyzing step indicates there is improvement in the amount and/or function of the pancreatic β-cells.

(12) A determining method for determining whether administration of a test substance has treated or relieved diabetes mellitus, comprising:

an administering step of administering a test substance to a biological sample that has incorporated

therein a recombinant expression vector in which a reporter gene that encodes an extracellular secreted product is placed under the control of a promoter region of a pancreatic β-cell-specific gene;

a detecting step of detecting the product of the reporter gene that is expressed in the biological sample that was administered with the test substance;

an analyzing step of analyzing an amount and/or function of pancreatic β-cells based on a result of detection performed in the detecting step; and

a determining step of determining that the administration of the test substance has treated or relieved diabetes mellitus, when a result of analysis in the analyzing step indicates there is improvement in the amount and/or function of the pancreatic β-cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram schematically illustrating a relevant structure of a plasmid construct used in Examples of the present invention.

Figure 2 is a diagram schematically illustrating a structure of pdx-1 gene promoter region used in Examples of the present invention.

Figure 3(a) is a diagram schematically illustrating a structure of a plasmid vector pIRES-hrGFP-1a used in

Examples of the present invention.

Figure 3(b) is a diagram schematically illustrating a structure of a plasmid vector pSEAP2-Basic used in Examples of the present invention.

Figure 4 is a diagram showing a result of SEAP activity measurement performed in Examples of the present invention.

Figure 5 is a diagram showing a result of experiment that determined how SEAP activity measurement was influenced by endogenous ALP in Examples of the present invention.

Figure 6 is a diagram showing a result of SEAP activity measurement performed on transgenic mice (13 lines and negative controls) in Examples of the present invention.

DESCRIPTION OF THE EMBODIMENTS

The present invention provides a method by which the amount and/or function of pancreatic β cells associated with the onset and development of diabetes mellitus is conveniently and accurately analyzed in real time, wherein the method detects the product of a reporter gene that is placed on the downstream side of a promoter region of a pancreatic β cell-specific gene. The invention also provides use of such methods. In the following,

description is made first as to an analyzing method of the present invention, followed by an analyzing kit and a screening kit as examples of use of such methods. Note that, recombinant expression vectors and transformants according to the present invention are closely related to the analyzing method, and as such they will be described in conjunction with the analyzing method.

[1] Analyzing Method

An analyzing method according to the present invention is not particularly limited as long as it analyzes the amount and/or function of pancreatic β cells with the use of a biological sample that has incorporated a recombinant expression vector in which a reporter gene is placed under the control of a promoter region of a pancreatic β cell-specific gene. As such, specifics of the method, such as steps, materials, and conditions, are not particularly limited.

As used in the invention, the "pancreatic β cell-specific gene" is preferably a gene that is specifically expressed in the β cells (B cells), which make up about 70% of the Islet of Langerhans (pancreatic Islet)—an endocrine gland that is found throughout the pancreas. However, it is not necessarily required that the gene is expressed specific to the pancreatic β cells as long as its expression is closely related to the amount and/or

function of the pancreatic β cells and monitoring (detection/evaluation) of the expression state of the gene allows the amount and/or function of the pancreatic β cells to be analyzed. Specific examples include mammalian transcription factors such as pdx-1 (also known as IPF1, STF-1, IDX-1, and XIHbox8) gene, (Ohlsson H et al., EMBO J 12: 4251-4259, 1993), NeuroD1 gene, Nkx2.2 gene, Nkx6.1 gene, Pax4 gene, and Pax6 gene. Other examples include insulin gene, glucokinase gene, GLUT2 gene, and amylin gene.

Among these pancreatic β cell-specific genes, pdx-1 gene is particularly preferable. It has been indicated that pdx-1 gene is commonly involved in the transcription of four kinds of pancreatic β cell-specific (characteristic) genes (insulin gene, glucokinase gene, GLUT2 gene, amylin gene), as well as somatostatin gene and HB-EGF gene. This has made pdx-1 gene a very important transcription factor. Involvement of pdx-1 gene in the early development of pancreas is also known. In this regard, there has been a report that a knock-out mouse (homo-deletion) lacks pancreas, and dies from diabetes at an early stage after birth (Jonsson J et al, Nature 371: 606-609, 1994).

The "promoter region" is also known as a transcription promoter, and it is a DNA region for

regulating initiation of gene transcription. As used herein, the "pancreatic β cell-specific gene promoter region" refers to promoter regions of the genes exemplified above, and its specific base sequence is not particularly limited. For example, mouse PDX-1 promoter (Acc. No.: NT_039324 REGION: 1408972 to 1418141) can be used, as will be described later in Examples.

Further, as used herein, the "recombinant expression vector in which a reporter gene is placed under the control of the promoter region" is not particularly limited in terms of a specific structure, as long as the promoter is so ligated and transferred into the vector as to express the reporter gene. Construction of the recombinant expression vector will be described later.

As used herein, the "reporter gene" refers to a gene that is used as an index of gene expression, with which gene expression can easily be detected *in vitro*, *ex vivo*, or *in vivo*. Examples of such reporter genes are structural genes of enzymes that catalyze the luminescent reaction or color reaction. Preferably, the reporter gene has such characteristics that it has (i) no background, (ii) an established method of sensitively and quantitatively detecting gene expression, and (iii) small effects on transformed cells, for example. Specific examples include: transposon Tn9-derived CAT (chlororamphenicol

acetyltransferase) gene; *E. coli*-derived β-D-galactosidase gene; *E. coli*-derived β-D-glucronidase (GUS) gene; firefly- or marine luminescence Vibrio-derived luciferase; *Aequorea coerulescens*-derived green fluorescence protein (GFP); jelly fish-derived aequorin; and alkaline phosphotase (ALP). In the Examples below, human extracellular secreted ALP (SEAP) was used.

Further, as used herein, the "biological sample having incorporated therein a recombinant expression vector" refers to a transformant that has incorporated a recombinant expression vector. The "biological sample" includes cells, tissues, organs, and animal individuals, for example. As will be described later in Examples, a method according to the present invention is applicable to transgenic animals. As such, the "transformant" includes not only transformed cells but also transgenic animals. As used herein, "animals" refers to mammals other than humans (non-human mammals), as well as other vertebrates. Preferable examples include various laboratory animals such as mice, rats, marmots, dogs, rabbits, monkeys, and chimpanzees.

For example, with transformed cells, tissues, and organs, it will be possible to perform an analyzing method of the present invention *in vitro* and *ex vivo*. When transformed laboratory animals (transgenic animals) are

used, an analyzing method of the present invention can be performed *in vivo*. Performing an analyzing method using transformed laboratory animals is highly useful because it allows for real time analyses of pancreatic β cell amount and/or pancreatic β cell function in living organisms.

Further, as used herein, the "pancreatic β cell amount" refers to the amount of β cell (or the number of β cells) in the pancreatic Islet. The "pancreatic β cell function" refers to the hormone secreting function of the pancreatic β cells, for example, such as the production and secretion of insulin, which is the most important metabolic hormone.

As described above, in an analyzing method of the present invention, the reporter gene is ligated on the downstream side of the promoter of genes that are specifically expressed in the pancreatic β cells. Thus, by monitoring the expression level of the reporter gene, the expression level of the pancreatic β cell-specific gene can be monitored. It is known that the expression level of pancreatic β cell-specific gene (for example, pdx-1 gene) increases with increase in the amount of pancreatic Islet cells (pancreatic β-cell amount). There has also been a report that a reduction in the level of insulin production in the pancreas under diabetic conditions (pancreatic β-cell glucotoxicity) leads to a reduction in the expression

level of the pancreatic β cell-specific gene (for example, pdx-1 gene). These facts are addressed in the following publications. (i) Possible Protection of Pancreatic β-Cell Against Glucose Toxicity DIABETES, VOL 48, P2398-2406, 1999, (ii) Glucose Toxicity in β-Cells: Type 2 Diabetes, Good Radicals Gone Bad, and the Glutathione Connection DIABETES, VOL 52, P581-587, 2003, (iii) Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants Proc. Natl. Acad. Sci. USA Vol. 96, P10857-10862 Medical Sciences, 1999, (iv) Beta-cell adaptation and decompensation during the progression of diabetes. DIABETES, VOL 50, P581-587, 2001, (v) Glycation-dependent, Reactive Oxygen Species-mediated Suppression of the Insulin Gene Promoter Activity in HIT Cells J. Clin. Invest. Volume 99, Number 1, P144-150 1997.

An analyzing method of the present invention therefore evaluates the expression level of a pancreatic β cell-specific gene through the reporter gene, and thereby analyzes and evaluates, in real time, fluctuations of pancreatic β-cell amount (or β-cell count), or conditions of insulin production (levels of insulin production) in the pancreatic β-cells. That is, the method analyzes pancreatic β-cell amount and/or pancreatic β-cell function.

Specific steps of an analyzing method according to the present invention include, for example, a transforming step, a detecting step (extracting step, extract detecting step), and an analyzing step. By an analyzing method including such steps, the amount and/or function of the pancreatic β-cells can be analyzed in real time, both conveniently and accurately. The analyzing method may additionally include an expression vector constructing step in which a recombinant expression vector is constructed. The following describes the respective steps.

[1-1] Expression Vector Constructing Step

An expression vector constructing step performed in the present invention is not particularly limited as long as the recombinant expression vector is constructed in which the reporter gene is placed under the control of a pancreatic β cell-specific gene promoter region. That is, in the expression vector constructing step, a recombinant expression vector is constructed in such a manner that the reporter gene is expressed under the control of the promoter. As such, the order of the sequences, the material of the vector, or the like can be suitably selected according to the type of host cell, tissue, organ, or animal, etc.

As the carrier vector of the recombinant expression vector, various types of conventional vectors can be used.

Some of the examples of such conventional vectors are plasmids, phages, and cosmids, and they can be suitably selected according to the type of host cell or the method of transfer. Specific examples are pBR322, pBR325, pUC19, pUC119, pBluescript, pBluescriptSK, and pBI vectors.

In addition to the promoter and reporter gene, the recombinant expression vector may include other DNA segments. Non-limiting examples of DNA segments are terminators, selection markers, enhancers, and base sequences for improving translation efficiency.

The terminator is not particularly limited and conventional terminators can be used as long as it serves as a transcription termination site. With the terminator suitably placed in the recombinant expression vector, the vector introduced into an animal cell does not cause syntheses of unnecessarily long transcripts, or there will be no reduction in the number of plasmid copies in the presence of a strong promoter.

As the selection marker, a chemical-resistant gene can be used, for example. Specific examples of chemical-resistant genes are those resistant to ampicillin, hygromycin, bleomycin, kanamycin, gentamicin, and chloramphenicol. With such chemical-resistant genes, transformants raised in an antibiotic culture medium can easily be screened for transformants that have

incorporated the recombinant expression vector. Further, as will be described later in Examples, other reporter genes such as GFP can be ligated on the downstream side of the reporter gene.

As the enhancer, a portion of the DNA base sequence is used that promotes transcription of neighboring genes that are located on the same deoxyribonucleic acid (DNA) strand (cis position). For example, the sequence with the recurring unit of 72 bases near the replication origin of monkey simian virus 40 (SV 40) can be used as the enhancer. Other than this example, conventional enhancer sequences can be used as well. Using such enhancers improves transcription activity even when the promoter region alone is not sufficient to induce strong expression of the reporter gene. This ensures that the product of the reporter gene is detected without fail. As described so far, the recombinant expression vector may contain various types of DNA segments depending on its purpose, the type of host cell used, etc.

The method of constructing the recombinant expression vector is not particularly limited. The promoter, the reporter gene, and optional other DNA segments are introduced in a predetermined order into a suitably selected carrier vector. For example, the reporter gene and the promoter (and optionally, an enhancer, a terminator,

etc.) are ligated to construct an expression cassette, which is then introduced into the vector.

In constructing the expression cassette, the order of DNA segments can be regulated by, for example, having complementary ends at the excision sites of each DNA segment, and then causing the reaction with a ligase. In the case where the expression cassette contains a terminator, the DNA segments are ordered such that the promoter, the reporter gene, and the terminator are placed in this order from the upstream side. In the case where the enhancer is contained in the expression cassette, the DNA segments are ordered such that the promoter, the enhancer, and the reporter gene are placed in this order from the upstream side. Further, the type of reagent used to construct the recombinant expression vector is not particularly limited. That is, the type of restriction enzyme or ligase is not particularly limited and may be suitably selected from commercially available products.

Further, the amplification (proliferation) method (producing method) of the recombinant expression vector is not particularly limited and conventional methods can be used. Generally, *E. coli* is used as a host, and the recombinant expression vector is amplified therein. In this case, the type of *E. coli* is suitably selected according to the type of vector used.

[1-2] Transforming Step

In the transforming step performed in the present invention, the recombinant expression vector is introduced into a biological sample to produce the transformant, and the reporter gene is expressed in the transformant.

The method by which the recombinant expression vector is introduced into the host cell (transforming method) is not particularly limited, and conventional method can be suitably used according to the type of host animal cell. Specifically, a method in which competent cells are artificially produced through treatment with calcium chloride, or a method in which the recombinant expression vector is directly introduced into the animal cell can be used, for example. As examples of a method by which the recombinant expression vector is directly introduced into the cell, the following methods are available: a micro injection method, an electroporation method, a polyethylene glycol method, a particle gun method, a protoplast fusion method, and a calcium phosphate method.

Suitable examples of host cells to which the recombinant expression vector is introduced include embryo stem cells, somatic stem cells, and somatic cells (bone marrow cells, liver cells, intestinal canal cells, etc.)

of mammals. Among these examples, the pancreatic β-cells and undifferentiated cells of the pancreas are particularly preferable. This is because the promoter on the recombinant expression vector is a promoter of the gene specific to the pancreatic β-cells, and because an object of the present invention is to analyze the amount and/or function of the pancreatic β-cells using the expression of the gene as an index.

In an analyzing method according to the present invention, the recombinant expression vector may be suitably selected according to the type of animal to be analyzed. Alternatively, a multi-purpose recombinant expression vector may be constructed in advance and introduced into animal cells. That is, an analyzing method according to the present invention may or may not contain the recombinant expression vector constructing step described in Section [1-1] above.

Further, the analyzing method of the present invention may be carried out by performing subsequent steps of the detecting step with the use of, if available, a commercially marketed analogue of the transformant obtained in the transforming step, or a transformant that has been separately prepared. This is not to be regarded as breaking the continuity of the sequence from the transforming step to the subsequent steps of the detecting

step. Rather, on macroscale, these steps should be regarded as a series of steps. In other words, the technical scope of the present invention does not exclude the case where the respective steps of the analyzing method are carried out at different times and different locations.

[1-3] Detecting Step

The detecting step carried out in the present invention is the step of detecting the product of the reporter gene expressed in the transformant described in Section [1-2]. The product of the reporter gene is mRNA or protein, for example. In the case where the reporter gene is a structural gene of an enzyme that catalyzes the luminescent reaction or color reaction reaction, it is more preferable that protein is detected.

As a method of detecting the product of the reporter gene, conventional methods can be suitably used according to the type of reporter gene. For example, when the reporter gene is GFP, the luminescence intensity of the GFP can be measured to quantitatively detect the product of the reporter gene. Further, when ALP is used as the reporter gene, the product of the reporter gene can be quantitatively detected by measuring ALP activity, as will be described later in Examples. In this case, detection can be conveniently carried out with the use of a commercially available ALP activity detecting kit, for

example.

When the gene product is mRNA, detection can be made by running quantitative RT-PCR. In this case, quantitative RT-PCR may be "real time PCR." Real time PCR can be performed by a conventional method such as an intercalater method, a TaqMan probe method, or a cycling probe method. In the case where quantitative PCR is not performed, detection most commonly follows the following procedure. First, a sample containing nucleic acids is electrophorased, and, after Southern blotting or Northern blotting, the sample is quantified with labeled probes. In the case where different kinds of nucleic acids are to be quantified at the same time, a DNA chip or a DNA microarray may be used with these methods, or without carrying out these methods.

In an analyzing method according to the present invention, the reporter gene is preferably a gene that encodes an extracellular secreted product. Examples of such reporter genes include a gene that encodes a protein that includes extracellular secreted signal peptides, and a gene that encodes a chimeric protein in which the signal peptides and reporter gene are fused together.

In this case, the protein (gene product) encoded by the reporter gene is secreted from the transformed cells, and therefore the product of the reporter gene can be

collected and detected by collecting the supernatant of the transformed cell culture, or the blood or urine of the transgenic animals, without damaging the cells or animals. Thus, in the case where the reporter gene is a gene that encodes an extracellular secreted product, the detecting step includes at least the extracting step and an extract detecting step, as described below.

[1-3-1] Extracting Step

The extracting step carried out in the present invention is the step of extracting an extract from the transformant described in Section [1-2] above. As used herein, "extracting an extract" refers to collecting an extract that contains the product of the reporter gene, when the biological sample is a cell, tissue, organ, or the like, or more specifically, collecting the supernatant of a cell or tissue culture, or the like, for example. In the case where the biological sample is an individual animal, "extracting an extract" refers to collecting animal's peripheral blood, peripheral body fluid, urine, or the like that includes the product of the reporter gene. As such, the reporter gene is preferably selected from extracellular secreted genes which encode products that are secreted in the culture supernatant, urine, or the like.

By the extracting step, the product of the reporter gene can be collected in real time, without damaging the

cells, tissues, or animal individuals.

A specific technique of extracting (collecting) the extract is not particularly limited, and conventional methods can be used. For example, in the case of collecting the supernatant of a cell culture, the extract can be collected by centrifugation, filtration, or the like. In the case of collecting blood, a syringe may be used to collect the blood. In the case of urine, collection can be made by simply collecting feces of the animal.

[1-3-2] Extract Detecting Step

The detecting step carried out in the present invention is the step of detecting a product of the reporter gene contained in the extract, which may be a culture supernatant, urine, blood, etc, as described in Section [1-3-1] above.

As to the step of detecting the product of the reporter gene in the extract, no explanation is given here because it can be performed in the same manner as the foregoing detecting step.

[1-4] Analyzing Step

The detecting step carried out in the present invention is the step of analyzing the amount and/or function of pancreatic β-cells based on the result of detection made in the detecting step described in Section [1-3] above. Specifically, after the quantitative detection of

the product of the reporter gene in the detecting step, the amount and/or function of the pancreatic β-cells is analyzed and evaluated based on the quantitative level. For example, in the analyzing step, the amount and/or function of the pancreatic β-cells is considered to have improved (recovered) if the quantitative level of the product of the reporter gene has increased. On the contrary, if the quantitative level of the product of the reporter gene has decreased, the amount and/or function of the pancreatic β-cells is considered to have deteriorated.

As used herein, the "analyzing step" can be regarded as the step of statistically analyzing the detection result (quantitative level) obtained in the detecting step. The step whereby the detection result is statistically analyzed can be performed by conventional methods, for example, such as a *t* test, or a non-parametric test.

[1-5] Other Steps, Other Methods

An analyzing method according to the present invention may include other steps, in addition to the transforming step and the recombinant vector constructing step. Specifically, the analyzing method may include a screening step of screening a transformed biological sample for suitable individuals.

The screening method is not particularly limited. For

example, screening may be made based on chemical resistance such as hygromycin resistance. Alternatively, the product of the reporter gene may be used as an index to perform screening, after culturing and growing the transformants.

The gist of the present invention is to provide a method for objectively analyzing (evaluating) the amount and/or function of pancreatic β-cells. As such, the present invention is not just limited to the respective procedures of transformation, extraction, detection, and analysis described above. Rather, it should be appreciated that the scope of the present invention also includes an analyzing method in which these procedures are not used.

[2] Use

With the foregoing advantages of the analyzing method according to the present invention, the invention can be used in a variety of ways. In the following, description is made as to an analyzing kit, a screening method, and a determination method, as exemplary use of the analyzing method according to the present invention.

[2-1] Analyzing Kit

An analyzing kit according to the present invention is for performing the analyzing method described in Section [1] above, and specific structures, materials, tools, and the like of the kit are not particularly limited. In

other words, the analyzing kit is not particularly limited as long as it is designed to perform the respective steps of the analyzing method.

For example, (a) in order to perform the transforming step, the recombinant expression vector is included in which the reporter gene is placed under the control of a promoter region of the pancreatic β cell-specific gene. (b) The analyzing kit may further include a transformant that has incorporated therein the recombinant expression vector of (a). This is more convenient because the transforming step will not be necessary. (c) For the transforming step, the analyzing kit preferably includes reagents for introducing the recombinant expression vector of (a) into animal cells. As used herein, "reagents" refers to various chemicals, experimental equipment and tools, etc. The reagents used for transformation can be selected from conventionally available reagents, and are not limited to a specific structure. Specific examples are enzymes and buffers, which are selected according to the type of transformation. Optionally, an experimental material such as a micro centrifugal tube may be included. The analyzing kit may further include reagents for preparing competent cells, and tools such as a heat block. (d) For the detecting step, the analyzing kit preferably includes reagents for detecting the product of the reporter

gene set forth in (a). The reagents and experimental equipment used for detection can be suitably combined according to the type of reporter gene.

The analyzing kit may further include, for example, various materials and reagents for performing the expression vector constructing step. Further, a tool such as a syringe may be included for collecting blood in the extracting step. For the detecting step, the analyzing kit may include, for example, various reagents, experimental equipment, and detecting tools that are required to detect the product of the reporter gene. For the analyzing step, various types of arithmetic devices (for example, computers) that are required for the analysis may be included.

With the analyzing kit of the foregoing structure, the analyzing method according to the present invention can be performed both conveniently and reliably.

[2-2] Screening Method

An analyzing method of the present invention is applicable to a method that determines the effectiveness of a potential drug substance in the drug screening that uses laboratory animals, etc. Specifically, a screening method according to the present invention includes: an administering step of administering a test substance to a biological sample that has incorporated therein the

recombinant expression vector; a detecting step of detecting a product of the reporter gene that is expressed in the biological sample that was administered with the test substance; an analyzing step of analyzing the amount and/or function of β-cells based on the result of detection in the detecting step; and a determining step of determining that the test substance is a potential substance of an anti-diabetic drug, when the result of analysis in the analyzing step indicates there is improvement in the amount and/or function of the β-cells. Notwithstanding the above, specific structures and conditions of the screening method are not particularly limited. That is, a screening method according to the present invention is not particularly limited as long as it uses an analyzing method of the present invention.

For example, in the screening method, if there is an increase in the quantitative level of the product of the reporter gene, it can be determined that the test substance has acted to raise the expression level of the pancreatic β cell-specific gene. As described above, there have been reports that the expression level of the pancreatic β cell-specific gene (for example, pdx-1 gene) increases with an increasing amount of pancreatic Islet cell (pancreatic β-cell amount), and that the expression level of the pancreatic β cell-specific gene (for example,

pdx-1 gene) decreases when there is a reduction in the level of insulin production in the pancreas under diabetic conditions (pancreatic β cell glucotoxicity). Therefore, the substance that increases the expression level of the pancreatic β cell-specific gene can be determined as a substance that increases the amount of pancreatic β-cell, and/or improves the function of pancreatic β-cells.

More specifically, by using a transformed cell or tissue as a biological sample, it is possible to screen for, either *in vitro* or *ex vivo*, a substance that increases the amount of pancreatic β-cell, or a substance (factor) that improves the function of pancreatic β-cells. Further, by using a transgenic animal as a biological sample, it is possible to screen for, *in vivo*, a substance that increases the amount of pancreatic β-cell, or a substance (factor) that improves the function of pancreatic β-cells.

That is, an analyzing method of the present invention can be used to conveniently and reliably screen for a potential substance of an anti-diabetic drug. As used herein, the "potential substance of an anti-diabetic drug" refers to any substance desired by an experimenter. Further, the "potential substance of an anti-diabetic drug" can be described as a substance that increases the amount of pancreatic β-cell, and/or improves the function of pancreatic β-cells.

[2-3] Determining Method

An analyzing method of the present invention is applicable to a method in which a test substance is administered to a laboratory animal, etc. to determine whether the test substance is effective in treating or relieving diabetes mellitus. Specifically, a determining method according to the present invention includes: an administering step of administering a test substance to a biological sample that has incorporated therein the recombinant expression vector; a detecting step of detecting a product of the reporter gene that is expressed in the biological sample that was administered with the test substance; an analyzing step of analyzing the amount and/or function of β-cells based on the result of detection in the detecting step; and a determining step of determining that the administration of the test substance has treated or relieved diabetes mellitus, when the result of analysis in the analyzing step indicates there is improvement in the amount and/or function of the β-cells. Notwithstanding the above, specific structures and conditions of the determining method are not particularly limited. That is, a determining method according to the present invention is not particularly limited as long as it uses an analyzing method of the present invention.

For example, in the determining method, if there is

an increase in the quantitative level in the product of the reporter gene, it can be determined that the potential substance has acted to raise the expression level of the pancreatic β cell-specific gene. Thus, when there is an increase in the expression level of the β cell-specific gene, it can be determined that the administration of the test substance has increased the amount of pancreatic β-cell, and/or improved the function of pancreatic β-cells. That is, it can be determined that diabetes mellitus has been treated or relieved.

More specifically, by using a transformed cell or tissue as a biological sample, it is possible to evaluate, either *in vitro* or *ex vivo*, whether diabetes mellitus has been treated or relieved. Further, by using a transgenic animal as a biological sample, it is possible to evaluate, *in vivo*, whether diabetes mellitus has been treated or relieved.

That is, an analyzing method of the present invention can be used to conveniently and reliably evaluate (determine) effectiveness of a test substance in treating or relieving diabetes mellitus. In other words, the invention can be used to conveniently and reliably evaluate effectiveness of food products or medicaments that are intended to reduce sugar level or relieve diabetes mellitus.

The following will describe embodiments of the

present invention in more detail by way of Examples. It should be appreciated that the invention is not just limited to the Examples described below, and various modifications are possible to details of the invention. Further, the invention is not limited to the foregoing embodiments, but may be altered by a skilled person within the scope of the claims. An embodiment based on a proper combination of technical means disclosed in different embodiments is encompassed in the technical scope of the present invention.

[Examples]

As is well known, pdx-1 gene activates expression of the pancreatic β cell-specific gene, and is a differentiation inducer indispensable for the development of pancreas. As examples of factors that affect the promoter activity or expression of pdx-1 gene, there has been a report that insulin, glucogan-like peptide-1 (GLP1), thyloid hormone, heparine binding epidermal growth-like factor (HB-EGF), and TNF-α raise the promoter activity of pdx-1 gene *in vitro* (Susan C, et al., Biochemical and Biophysical Research Communications 2002, 277-284). In another report, administration of retinoic acid is said to raise the expression level of pdx-1 gene *in vitro* (Sidhartha Singo Tulachan, et al. DIABETES 2003, 76-84). Further, it has been reported that GLP-1 raises the expression level of

pdx-1 protein *in vitro* (J. Buteau, et al., Diabetologia 1999, 856-864).

While many reports indicate that various substances can be used to raise the expression level of pdx-1 gene *in vitro*, there has been no report of substances that increase the expression level of pdx-1 gene *in vivo*. Further, no method has been developed that conveniently analyzes in real time fluctuations of pdx-1 gene expression.

In order to develop a method by which changes in the expression level of pdx-1 gene is conveniently analyzed in real time, and thereby analyze changing expression levels of pdx-1 gene *in vivo*, a transgenic mouse was constructed in which the reporter gene is placed under the control of the promoter of pdx-1 gene. Specific procedures are as follows.

(1) Construction of Plasmid Constructs

First, a plasmid construct was prepared in which human secreted alkaline phosphatase (SEAP) was placed under the control of the promoter region of pdx-1 gene. Specifically, from the genomic DNA obtained from the spleen of C57BL6 mouse, replicates of the promoter region of pdx-1 gene (mouse PDX-1 promoter Accession #: NT_039324 REGION: 1408972 to 1418141) were prepared. The replicates were prepared to -6.5 kb upstream of the ORF of pdx-1, so as to include all of regions I, II, III, IV.

The following primers were used for the procedure. The replicates were sequenced after the procedure.

- Primer PDX1pro_f_Hind3_Spe1:

5'-ACCCAAGCTTGACTAGTCAGGATCCAGGTTTA-3'

- Primer PDX1pro_r_Pst1:

5'-GTGTGTGTGAGTCTATTCTCAACTGCA-3'

- Primer PDX1pro_f_Mlu1_Pst1:

5'-GGACGACGCGTGACTAGTCCTCCAACATCAGACGTG CAC-3'

- Primer PDX1pro_r_Sma1_Not1:

5'-AAAAGGAAAAGCGGCCGCAGCCCCGGGTCGGAGCT ACAA-3'

Next, the CMV promoter region of plasmid vector pIRES-hrGFP-1a (BD Biosciences) shown in Figure 3(a) was excised with restriction enzymes XbaI and NotI. The replicated promoter region of pdx-1 gene was then inserted between XbaI and NotI.

Thereafter, plasmid vector pSEAP2-Basic (BD Biosciences, GenBank Accession #: U89937) shown in Figure 3(b) was treated with restriction enzymes EcoRI and BsmI to obtain fragments that include SEAP structural gene. The SEAP fragment was then inserted between EcoRI and SalI at the multiple cloning site (MCS) of plasmid vector pIRES-hrGFP-1a shown in Figure 3(a). As a result, plasmid construct pPDX1pro-SEAP-IRES-GFP

was obtained.

(2) Functional Analysis of pPDX1pro-SEAP-IRES-GFP *in vitro*

Next, a functional Analysis of pPDX1pro-SEAP-IRES-GFP was made *in vitro*. Specifically, the activity of secreted ALP (SEAP activity) that was expressed under the control of the promoter of pdx-1 gene was measured *in vitro*. More specifically, pPDX1pro-SEAP-IRES-GFP was introduced into a β-cell line and a non β-cell line to obtain transformants. As a negative control, plasmid p-promoterless-SEAP-IRES-GFP was constructed that lacked the promoter of the pPDX1pro-SEAP-IRES-GFP. Plasmid p-promoterless-SEAP-IRES-GFP was also introduced into a β-cell line (M6) and a non β-cell line (HepG2) to obtain transformants.

After 72 hours, alkali phosphatase (ALP) activity of the transformants was measured. The measurement was made with a Chemiluminescent SEAP Assay Kit (BD Bioscience) according to the manufacturer's protocol. The results are shown in Figure 4. In Figure 4, "PDX1-SEAP-GFP" is the transformant with pPDX1pro-SEAP-IRES-GFP, and "promoterless-SEAP-GFP" is the transformant with p-promoterless-SEAP-IRES-GFP.

As shown in Figure 4, the β-cell line that had

incorporated pPDX1pro-SEAP-IRES-GFP had an ALU activity RLU278687, and the non β-cell line that had incorporated pPDX1pro-SEAP-IRES-GFP had an ALU activity RLU61098. The negative controls, the β-cell line and non β-cell line with p-promoterless-SEAP-IRES-GFP, had RLU77610 and RLU69298, respectively. The result therefore showed that pPDX1pro-SEAP-IRES-GFP was specifically expressed only in the pancreatic β-cells, proving that pPDX1pro-SEAP-IRES-GFP was indeed effective.

(3) Influence of Endogenous ALP activity

Next, assessment was made as to the influence of endogenous ALP activity on the SEAP Assay performed in Section (2) above. Specifically, in order to find how the measurement of SEAP activity was influenced by the endogenous ALP in the non-β-cell line (HepG2) and mouse blood plasma, assessment was made as to whether the incubation performed at 65°C for 30 minutes during the procedure of the Chemiluminescent SEAP Assay Kit would eliminate the activity of the endogenous ALP in the non-β-cell line (HepG2) and mouse blood plasma.

The results are shown in Figure 5. In Figure 5, "plasma ALP1" and "plasma ALP2" are endogenous ALP in the mouse blood plasma, and "HepG2 ALP" is endogenous ALP in the non-β-cell line (HepG2).

As shown in Figure 5, by the incubation performed at 65°C for 30 minutes, all of the endogenous ALP in the non-β-cell line (HepG2) and mouse blood plasma lost their activities. It was therefore confirmed that the endogenous ALP activity has absolutely no effect on the Chemiluminescent SEAP Assay performed in Section (2) above. In other words, it can be said that the results obtained in Section (2) are the results of measurement that only measured the activity of secreted ALP (SEAP) that is expressed under the control of the promoter of pdx-1 gene.

(4) Functional Analysis of pPDX1pro-SEAP-IRES-GFP *in vivo*

(4-1) Preparation of pPDX1pro-SEAP-IRES-GFP Transgenic Mouse

With the pPDX1pro-SEAP-IRES-GFP constructed in Section (1) above, a pPDX1pro-SEAP-IRES-GFP transgenic mouse was produced. The actual procedure was performed by Japan SLC Inc., Hamamatsu, Japan, a company specialized in producing transgenic mice. Mice of 24 lines were provided by SLC Inc. These mice were genotyped, and 13 lines (may be referred to as Line B, C, D, F, G, H, I, J, K, L, M, N, O hereinafter) out of 24 lines were transgene positive. As negative controls, sibling mice without transgene (may be referred to as "SEAP(-)" hereinafter)

were used.

(4-2) Functional Analysis of pPDX1pro-SEAP-IRES-GFP

A functional analysis of pPDX1pro-SEAP-IRES-GFP was made in the transgenic mice. A measurement of SEAP activity was made in the 13 lines that were transgene positive in Section (4-1). SEAP activity was measured according to the following procedures.

- Measurement Method of SEAP activity

Measurement was made with the Chemiluminescent SEAP Assay Kit (BD Bioscience).

(i) Sample Preparation

1. Twenty micro liters of blood was mixed with 180 μl of PBS. After centrifugation, the supernatant was separated.
2. One-Hundred Sixty micro liters of supernatant was placed in a new tube (and preserved at -20°C if needed).

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(ii) Chemiluminescent SEAP Assay

1. Excess amounts of Chemiluminescent Enhancer and Assay Buffer were allowed to stand at room temperature.
2. A required amount of 1 × Dilution Buffer (appended 5 × Dilution Buffer:dH_2O=1:4)

was prepared and allowed to stand at room temperature.

3. Twenty micro liters of dissolved sample was placed in a transparent 0.5 ml tube.
4. Thirty micro liters of 1 × Dilution Buffer was added and thoroughly mixed in the tube prepared in Step 3.
5. The sample prepared in Step 4 was allowed to stand at 65°C for at least 30 minutes (inactivation of endogenous ALP).
6. The sample prepared in Step 5 was cooled to room temperature on ice.
7. Fifty micro liters of Assay Buffer was added to each sample, and the mixture was allowed to stand at room temperature for 5 minutes.
8. CSPD (1.25 mM) was prepared (appended 25 mM CSPD:Chemiluminescent Enhancer = 1:19)
9. Fifty micro liters of CSPD prepared in Step 8 was added to each sample and allowed to stand at room temperature for 5 minutes.
10. Measurement was made within 10 to 60 minutes after the addition of CSPD.

(iii) Results

Figure 6 shows the results of SEAP activity

measurement on transgene positive Lines B, C, D, F, G, H, I, J, K, L, M, N, O. It can be seen from Figure 6 that the SEAP activity was much stronger in all of the lines as compared with the negative control SEAP(-). This confirmed that the pPDX-1pro-SEAP-IRES-GFP was indeed effective also *in vivo*.

The foregoing results showed that an analyzing method according to the present invention could also be implemented *in vivo*, using the transgenic mice.

INDUSTRICAL APPLICABILITY

An analyzing method according to the present invention detects expression of a reporter gene placed on the downstream side of a promoter region of a pancreatic β cell-specific gene, and thereby indirectly monitors the expression level of the pancreatic β cell-specific gene. It is known that the expression level of pancreatic β cell-specific gene (for example pdx-1 gene) rises when there is an increase or improvement in the amount or function of pancreatic β cells. Thus, by monitoring the expression level of pancreatic β cell-specific gene by the analyzing method according to the present invention, it is possible to analyze the amount and/or function of pancreatic β-cells in real time, both conveniently and accurately. An analyzing method according to the present

invention is therefore applicable to, for example, a screening method of anti-diabetic drugs, or a method of evaluating or accessing the effectiveness of diabetes treatment.

As described above, with an analyzing method according to the present invention, the amount or function of pancreatic β-cells can be analyzed in real time, both conveniently and accurately. The invention is therefore useful in the development of drugs and methods for treating or diagnosing diabetes mellitus. Further, with the invention, functions of health foods or medicaments that are claimed to be effective for the treatment of diabetes mellitus can be evaluated. The invention is therefore applicable to a wide variety of industrial fields, including, for example, medical industry and food industry.